

# ***Coptotermes gestroi* (Isoptera: Rhinotermitidae) in Brazil: possible origins inferred by mitochondrial cytochrome oxidase II gene sequences**

**C. Martins, L.R. Fontes, O.C. Bueno, and V.G. Martins**

**Abstract:** The Asian subterranean termite, *Coptotermes gestroi*, originally from northeast India through Burma, Thailand, Malaysia, and the Indonesian archipelago, is a major termite pest introduced in several countries around the world, including Brazil. We sequenced the mitochondrial COII gene from individuals representing 23 populations. Phylogenetic analysis of COII gene sequences from this and other studies resulted in two main groups: (1) populations of Cleveland (USA) and four populations of Malaysia and (2) populations of Brazil, four populations of Malaysia, and one population from each of Thailand, Puerto Rico, and Key West (USA). Three new localities are reported here, considerably enlarging the distribution of *C. gestroi* in Brazil: Campo Grande (state of Mato Grosso do Sul), Itajaí (state of Santa Catarina), and Porto Alegre (state of Rio Grande do Sul).

**Key words:** *Coptotermes gestroi*, cytochrome oxidase II, termite, introduced species, Brazil.

**Résumé :** Le termite souterrain *Coptotermes gestroi*, originaire du nord-est de l'Inde et présent en Birmanie, Thaïlande, Malaisie et l'archipel indonésien, est un insecte nuisible introduit dans plusieurs pays du monde dont le Brésil. Les auteurs ont séquencé le gène mitochondrial COII chez des individus représentant 23 populations. L'analyse phylogénétique des séquences COII issues de cette étude et d'autres études antérieures a permis de former deux groupes principaux : (1) populations de Cleveland (É-U) et quatre populations de la Malaisie et (2) populations du Brésil, quatre populations de la Malaisie, une de la Thaïlande, une de Porto Rico et une de Key West (É-U). Trois nouvelles localités sont rapportées ici et augmentent considérablement l'aire de distribution du *C. gestroi* au Brésil : Campo Grande (état du Mato Grosso do Sul), Itajaí (état de Santa Catarina) et Porte Alegre (état de Rio Grande do Sul).

**Mots-clés :** *Coptotermes gestroi*, cytochrome oxydase II, termite, espèce introduite, Brésil.

[Traduit par la Rédaction]

## **Introduction**

*Coptotermes gestroi* is the most important pest among the species of termites present in urban areas of Brazil. The species is native in the Oriental Region (see the taxonomic revision by Kirton and Brown (2003), which assigns the previous denomination *C. havilandi* as a junior synonym of *C. gestroi*). Its introduction history, distribution, and complex infestation dynamics in South America were reviewed by Fontes and Milano (2002) and Milano and Fontes (2002a, 2002b).

Since its introduction in Brazil, *C. gestroi* has clearly been expanding its geographic range in a discontinuous way restricted to urban areas. This termite was initially discovered in infested buildings in 1923 and 1934 in the cities of Rio de Janeiro and Santos, respectively. Both cities are in the Southeast Region and have harbors that are economi-

cally important routes to the inland regions of the country. Because nests were hidden in the deep structures of ships and not in parts able to be landed, specimens of *C. gestroi* were off-loaded from infested ships by means of swarming alates from the mature nests or by means of young colonies (also called incipient colonies, since they are composed of the royal pair installed in a well-insulated chamber appropriate for setting up the eggs and larvae) recently installed in cargo transported to the mainland. Most likely this happened during the second half of the 19th century. Distant introductions, from city to city, in the subsequent decades have been caused by human transportation of young colonies, while within each city dispersal occurs mainly in a continuous way from the original introduction point through swarming from the mature colonies, although the transportation of young colonies in cargos is also involved in the formation of secondary urban focuses of the pest.

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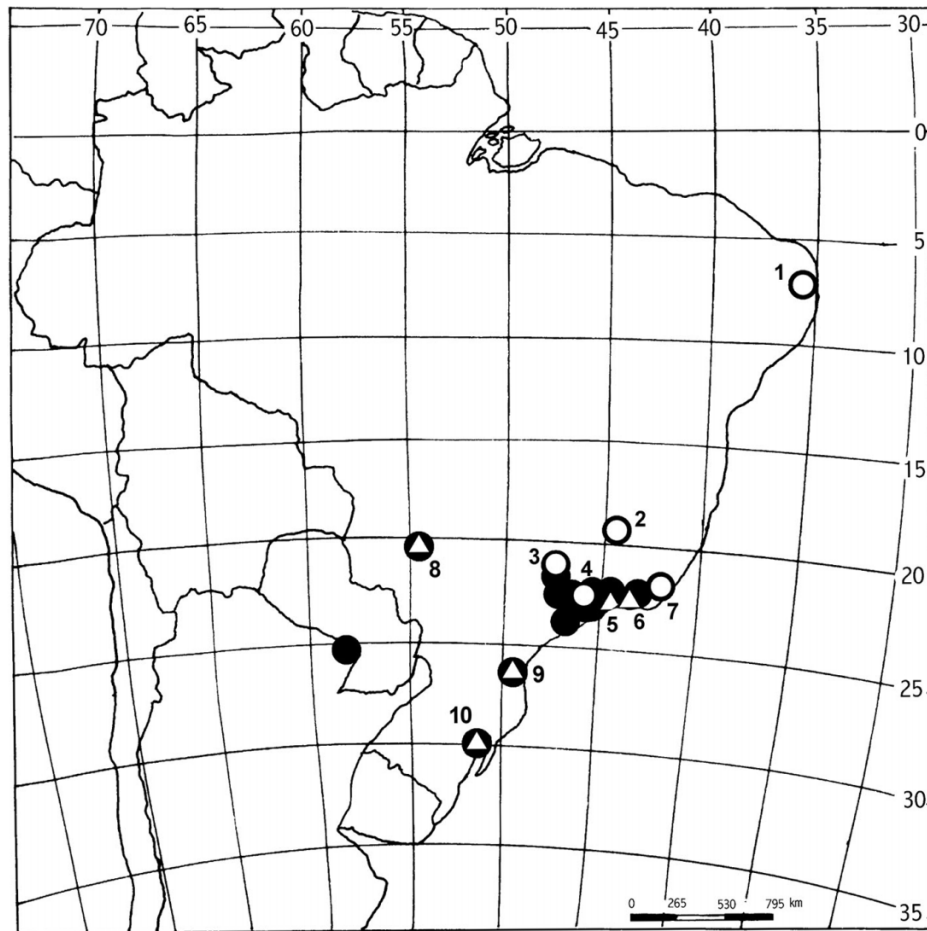
**C. Martins.** Universidade Federal do Piauí, Campus Ministro Reis Veloso, Biologia, Avenida São Sebastião, 2819, Reis Veloso, Parnaíba, PI 64202-020, Brazil.

**L.R. Fontes.** Rua Loefgreen, 1543, 104, São Paulo, SP 04040-032, Brazil.

**O.C. Bueno and V.G. Martins.**<sup>1</sup> Universidade Estadual Paulista Julio de Mesquita Filho, Centro de Estudos de Insetos Sociais, Avenida 24A, 1515, Rio Claro, SP 13506-900, Brazil.

<sup>1</sup>Corresponding author (e-mail: martv@uol.com.br).

**Fig. 1.** Distribution of *Coptotermes gestroi* in South America (adapted from Fig. 2 in Fontes and Milano 2002) and populations studied in this paper: 1, Recife; 2, Belo Horizonte; 3, Rio Claro; 4, São Paulo (major urban area in the continent); 5, São Sebastião; 6, Parati; 7, Rio de Janeiro; 8, Campo Grande; 9, Itajaí; 10, Porto Alegre. Open circles are previously reported localities. Open triangles (5, 6, 8, 9, 10) are new localities, reported for the first time in this paper, that enlarge the previously reported geographic distribution of the species. The locality in the far west is Asuncion, Paraguay.



Introductions due to transport of nests or infested pieces of wood or other material are not likely because (1) nests are large and thus easily detected and discarded, or die quickly if exposed to environmental conditions (it is even difficult to keep a large nest in laboratory conditions); (2) the species builds polycalyc nests and the royal pair quickly flee from the disturbed nest; and (3) neoteny, a phenomenon common to other pest termites, is extremely rare in *C. gestroi* populations in Brazil and is unlikely to be a prominent dispersion force, so fragmented populations (even if they are composed of thousands of termites) are condemned to death. Ornamental trees scattered along streets and in parks have been important reservoirs for *C. gestroi*. From these reservoirs termites swarm and continually infest or reinfest nearby buildings. The topology of rural and semirural areas, with natural vegetation, pastures, and patches of dense forest, serves to disrupt the pest's dispersal.

The termite is now distributed in cities along the Atlantic coast of Brazil, from Recife in the northeast to the cities of Itajaí and Porto Alegre in the south (new localities presented in this study), and has penetrated towards the west of the continent to reach the cities of Campo Grande in Brazil

(new locality presented in this study) and Asuncion in Paraguay (Fontes and Milano 2002, Fig. 1). Infestations in the northeastern, southern, and western regions of Brazil are recent (imported in the last few decades) and still restricted to a few districts in the infested cities.

The dispersal rate of *C. gestroi* in Brazil seems to have accelerated in the last few decades and the termite clearly presents a risk to cities in other regions of Brazil and all other countries in South America, all of which share common conditions for the establishment of this pest that interacts largely and successfully with the urban ecosystem, influenced by building patterns, urban trees, and control measures (Milano and Fontes 2002a, 2002b).

The discontinuous distribution of *C. gestroi* raises the question of how many independent introductions occurred from colonies coming from other regions of the world. All the populations in the Southeast Region of Brazil probably spread from the original stock introduced in the last half of the 19th century in the harbor cities of Rio de Janeiro and Santos, reaching by human transport the western cities of the continent. The cities of Recife in the northeast and Itajaí and Porto Alegre in the south are distant from the previous

**Table 1.** Collection sites of *Coptotermes gestroi* included in this study, Brazil.

Population in Fig. 1	Sample	City/district	State	Latitude (S)	Longitude (W)
1	E 1700	Recife	Pernambuco	08°05'07"	34°54'53"
2	E 1448	Belo Horizonte	Minas Gerais	19°56'07"	43°56'22"
3	E 693	Rio Claro	São Paulo	22°24'45"	47°33'46"
	E 697	Rio Claro	São Paulo	22°23'43"	47°32'33"
	E 719	Rio Claro	São Paulo	22°23'14"	47°33'50"
	E 1040	Rio Claro	São Paulo	22°23'45"	47°33'44"
	E 792	São Paulo / Ibirapuera	São Paulo	23°35'22"	46°39'37"
	E1047	São Paulo / Ibirapuera	São Paulo	23°35'21"	46°39'33"
	E 753	São Paulo / Jardim Bonfiglioli	São Paulo	23°34'32"	46°45'40"
	E 791	São Paulo / Lapa	São Paulo	23°31'29"	46°42'21"
	E 1049	São Paulo / Lapa	São Paulo	23°31'24"	46°42'30"
	E 752	São Paulo / Morumbi	São Paulo	23°35'42"	46°42'33"
4	E 790	São Paulo / Parque Continental	São Paulo	23°32'29"	46°45'40"
	E 1048	São Paulo / Parque Continental	São Paulo	23°32'39"	46°45'35"
	E 789	São Paulo / Praça da Sé	São Paulo	23°33'02"	46°34'59"
	E 1046	São Paulo / Praça da Sé	São Paulo	23°33'02"	46°34'59"
	E 751	São Paulo / Vila Clementino	São Paulo	23°28'50"	46°37'42"
	E 1447	São Sebastião	São Paulo	23°45'40"	45°24'50"
5	E 1104	Parati	Rio de Janeiro	22°54'33"	43°12'53"
6	E 1834	Rio de Janeiro	Rio de Janeiro	22°57'42"	43°12'57"
7	E 1103	Campo Grande	Mato Grosso do Sul	20°26'30"	54°38'52"
8	E 1137	Itajaí	Santa Catarina	27°39'73"	48°39'43"
9	E 1755	Porto Alegre	Rio Grande do Sul	30°03'42"	51°13'21"

introduction areas and are important coastal harbor cities that could have had independent introductions from the Oriental Region or other regions of the world.

Mitochondrial DNA, which is maternally inherited, is a rapidly evolving genome (Avisé 1994). The cytochrome oxidase gene is the most widely used mitochondrial gene for biological studies in insects (Simon et al. 1994). Mitochondrial DNA has also been used to investigate gene flow in termites (Jenkins et al. 1998, 1999, 2001, 2002). To track the possible origin of the Brazilian *C. gestroi* populations, we collected or received from researchers and pest control operators 23 samples. The cytochrome oxidase I (COI) and tRNA<sup>Leu</sup> genes and a portion of the cytochrome oxidase II (COII) gene were PCR amplified and sequenced and then compared with sequences available from GenBank to infer their relationships by phylogenetic analysis.

## Materials and methods

### Collection and identification

Samples consisted of workers and soldiers from 23 sites in Brazil (see Table 1 and Fig. 1), collected by pest control operators and local biologists. Termites were identified as *C. gestroi* by L.R. Fontes.

### DNA extraction, amplification, purification, and sequencing

Total DNA was extracted from single workers preserved in 80% ethanol. The dissociated tissues were incubated in a cell lysis solution (100 mmol/L Tris, pH 9.1, 100 mmol/L NaCl, 50 mmol/L EDTA, 0.5% SDS) at 55 °C for 3 h; protein residues were subsequently precipitated with 5 mol/L NaCl and centrifuged at 16 000g for 5 min. The supernatant

was transferred to a fresh 1.5 mL tube containing 600 µL of 100% isopropanol and then mixed gently by inversion and centrifuged at 16 000g for 5 min. The supernatant was removed and 600 µL of 70% ethanol was added to the tube; the contents were mixed gently by inversion and centrifuged at 16 000g for 3 min. The ethanol was removed and the pellet was dried for 10 min in a SpeedVac. DNA was resuspended in 30 µL of TE (10 mmol/L Tris, 1 mmol/L EDTA, pH 8).

An approximately 930 bp portion of the mitochondrial genome that includes a portion of the cytochrome oxidase I (COI) gene, the complete tRNA<sup>Leu</sup> gene, and a portion of the cytochrome oxidase II (COII) gene was amplified by PCR using the two mtDNA primers Geo C1-J-2792 (5'-ATACCTCGACGTTATTTCAGA-3') and Eva TK-N-3772 (5'-GAGACCATTACTTGCTTTCAGTCATCT-3') (Simon et al. 1994). PCR was performed in 25 µL volumes containing 250–500 ng of DNA template, 10 pmol of each primer, and Ready-To-Go mix (Amersham Pharmacia Biotech) in an MJ Research PTC-200 thermocycler. Amplifications were performed with the following cycling profile: 1 cycle of 10 min at 94 °C; 35 cycles of 94 °C for 1 min, 52 °C for 1 min and 20 s, and 68 °C for 2 min and 30 s; and 1 cycle of 10 min at 65 °C. The amplified PCR products were purified using the Wizard PCR Prep DNA Purification System (Promega, Madison, Wisconsin) and directly used in sequencing reactions with ABI BigDye Terminator chemistry (version 2.0; Applied Biosystems Inc., Foster City, California) following the manufacturer's protocol. Together with the above reported primer pair, an internal primer designed for this paper, PFN (sense) (5'-CTAACGTGGCAGATAAGTGC-3'), was also used for the sequencing reactions. The products of sequencing reactions were purified with

**Table 2.** GenBank accession number, species, locality, and source of the sequences used in phylogenetic analysis.

GenBank acc. No.	Species	Locality	Source
AY536408	<i>Coptotermes gestroi</i>	Malaysia: Penang Island	W.M. Ye et al., unpublished
AJ854171	<i>C. gestroi</i>	Malaysia	S. Cheng et al., unpublished
EF092290	<i>C. gestroi</i>	Thailand: Royal Forest Dept, Bangkok	Jenkins et al. 2007
DQ923416	<i>C. gestroi</i>	Malaysia	Jenkins et al. 2007
DQ923417	<i>C. gestroi</i>	Malaysia	Jenkins et al. 2007
DQ923418	<i>C. gestroi</i>	Puerto Rico	Jenkins et al. 2007
DQ923420	<i>C. gestroi</i>	USA: Cleveland, Ohio	Jenkins et al. 2007
EF092291	<i>C. gestroi</i>	USA: Key West, Florida	Jenkins et al. 2007
DQ923419	<i>C. gestroi</i>	Malaysia	Jenkins et al. 2007
AY683220	<i>Coptotermes formosanus</i>	USA: Cairo, Georgia	T.M. Jenkins et al., unpublished
AJ854175	<i>C. gestroi</i>	Malaysia	S. Cheng et al., unpublished
AJ854174	<i>C. gestroi</i>	Malaysia	S. Cheng et al., unpublished
AJ854173	<i>C. gestroi</i>	Malaysia	S. Cheng et al., unpublished
AF149739	<i>Cornitermes cumulans</i>	Brazil: Rio Claro, São Paulo	C. Martins et al., unpublished
AF149746	<i>Cornitermes bequaerti</i>	Brazil: Bauru, São Paulo	C. Martins et al., unpublished

95% isopropanol and 70% ethanol and run on an ABI PRISM 377 sequencer. All mtDNA amplicons were sequenced in both directions.

### Phylogenetic analysis

Sequences were edited by eye. Corresponding sequence pairs originating from primers C1-J-2792 and TK-N-3772 and from the internal primer PFN were aligned with the Clustal W option of BioEdit software (<http://www.ctu.edu.vn/~dvxe/Bioinformatic/Software/BioEdit.htm>) and then combined into a single consensus sequence. This sequence was 933 bp long, and 602 bases were used for comparisons with GenBank-derived sequences. Phylogenetic analyses were conducted using Bayesian inference and maximum parsimony. For Bayesian inference, the appropriate models for sequence evolution were estimated using the program MrModeltest v.2 (Nylander 2004). The appropriate model was found to be GTR+G (general time-reversible model). The Bayesian inference analyses were performed using MrBayes 3.0 (Huelsenbeck and Ronquist 2001). One million trees were generated, with a sample frequency of 100. Ten percent of the trees were discarded and posterior probabilities were calculated with the remaining trees. The sequences were analyzed by maximum parsimony using PAUP version 4.0b10 (Swofford 2003). Bootstrap support was calculated with 1000 replicates.

In the analysis, the tree was rooted using *Cornitermes cumulans* (GenBank accession number AF149739) and *Cornitermes bequaerti* (AF149746). Table 2 shows the GenBank sequences used as ingroup and outgroup sequences in the phylogenetic analyses.

### Results

The 23 populations of *C. gestroi* analyzed showed the same 933 base pairs of the mtDNA targeted: 135 bp corresponding to the partial COI gene, 23 bp corresponding to an intergenic spacer, 68 bp corresponding to the tRNA<sup>Leu</sup> gene, and 706 bp corresponding to the COII gene. Of the 933 nucleotides, 62% are A and T nucleotides and 38% are G and

C nucleotides, corroborating the high A+T frequencies for insects discussed in Simon et al. (1994).

The sequence was entered in GenBank with accession number GQ994994. A fragment of the COII gene (602 bp) from our data and from the GenBank sequences shown in Table 2 was analyzed by Bayesian inference using MrBayes (Huelsenbeck and Ronquist 2001) and maximum parsimony using the software PAUP 4.0b10 (Swofford 2003).

The Bayesian and maximum parsimony analyses recovered trees virtually identical in topology. The resulting tree shown in Fig. 2 was generated by MrBayes and suggests that the analyzed populations are clustered in two main groups. One group includes individuals collected in Cleveland (USA, GenBank accession number DQ923420) and other individuals from Malaysian collections (DQ923419, AJ854173, AJ854174, and AJ854175); these individuals share a transition mutation at position 201 of the COII gene (see Table 3). The second group constitutes a basal clade that includes the populations of Brazil, four populations of Malaysia (AY536408, AJ854171, DQ923416, and DQ923417), and the populations from Thailand: Bangkok (EF092290), Puerto Rico (DQ923418), and USA: Key West (EF092291), which share a transition mutation at position 354 of the COII gene (see Table 3).

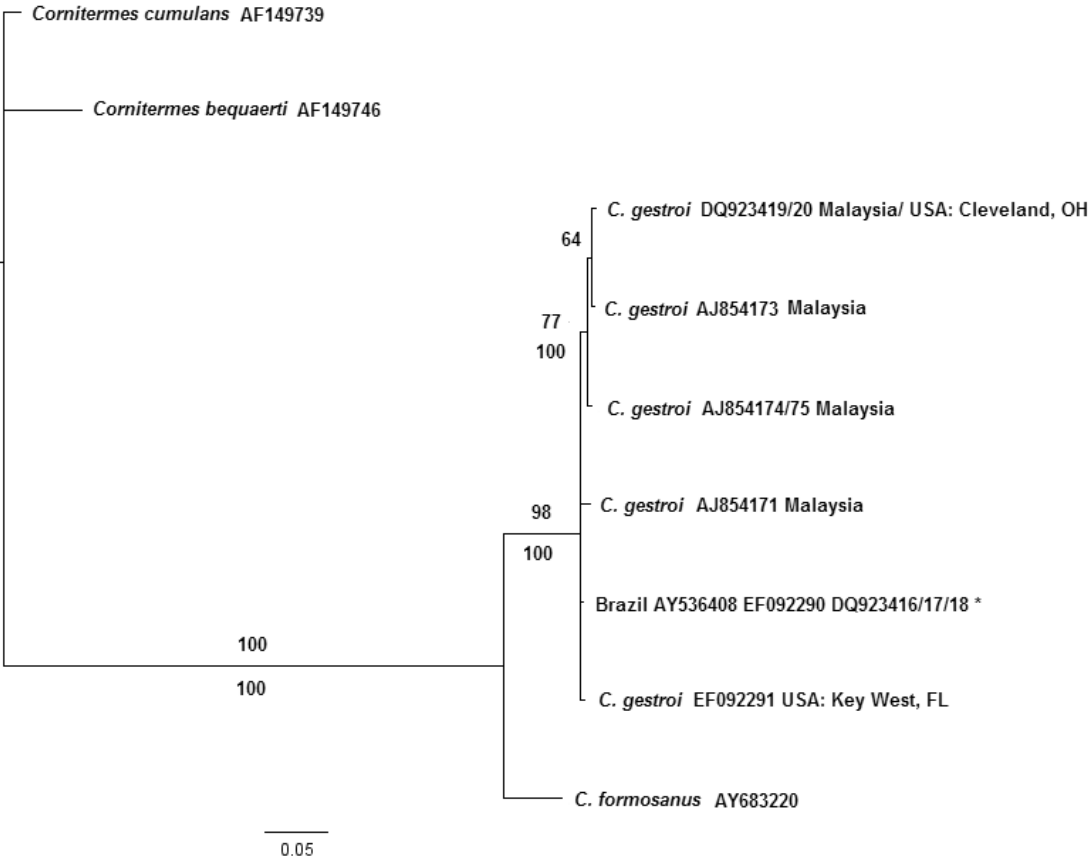
### Discussion

The COII genes have been used in phylogenetic analyses in several subterranean termite studies (Kambhampati et al. 1996; Austin et al. 2002; Jenkins et al. 2002; Ohkuma et al. 2004; Szalanski et al. 2004). In this paper the mtDNA sequences were used to determine the origins and genetic variability of *C. gestroi* populations introduced in Brazil.

This study suggests that *C. gestroi* populations in Brazil represent invasions from Malaysia and Thailand (Fig. 2). There appeared to be an introduction of Asian termites into the southeast regions of Brazil in the second half of the 19th century, supporting previous data in the literature (see review by Fontes and Milano 2002). Passive human transport through trade in subsequent decades dispersed *C. gestroi* to other Brazilian cities.



**Fig. 2.** Phylogenetic tree based on sequences from the cytochrome oxidase II (COII) gene. The Bayesian and maximum parsimony (MP) analyses recovered trees virtually identical in topology. The tree shown was generated by MrBayes. Numbers above branches are posterior probability values from Bayesian analyses. Numbers below branches are bootstrap support from MP analyses. AY536408, Malaysia: Penang Island; EF092290, Thailand: Bangkok; DQ923416/17, Malaysia; DQ923418, Puerto Rico. Bootstrap values below 60 are not shown.



**Table 3.** Variable sites in the mitochondrial cytochrome oxidase II gene of *Coptotermes gestroi*.

Sequence	Base number												
	201	206	211	216			346	351	356				
	.. ... ... ... ... ..... ... ... ...												
	201								354				
AF149739 <i>C.c.</i>	AT	<b>T</b> GCACCGCAATCATTTTA	.....	AAAGCTTGAGTT	<b>C</b> GACTCAT								
AF149746 <i>C.b.</i>	AT	<b>T</b> GCACCAGCAATTATTTTA	.....	AAAACCTGAATT	<b>T</b> GACTCAT								
DQ923420 <i>C.g.</i> Ohio	AT	<b>T</b> GCACCAGCAATCATCCTT	.....	TAAACTAGAATT	<b>T</b> GACTCAT								
DQ923419 <i>C.g.</i> Malaysia	AT	<b>T</b> GCACCAGCAATCATCCTT	.....	TAAACTAGAATT	<b>T</b> GACTCAT								
AJ854173 <i>C.g.</i> Malaysia	AT	<b>C</b> GCACCAGCAATCATCCTT	.....	TAAACTAGAATT	<b>T</b> GACTCAT								
AJ854175 <i>C.g.</i> Malaysia	AT	<b>C</b> GCACCAGCAATCATCCTT	.....	TAAACTAGAATT	<b>T</b> GACTCAT								
AJ854174 <i>C.g.</i> Malaysia	AT	<b>C</b> GCACCAGCAATCATCCTT	.....	TAAACTAGAATT	<b>T</b> GACTCAT								
Brazil	AT	<b>T</b> GCACCAGCAATCATCCTT	.....	TAAACTAGAATT	<b>C</b> GACTCAT								
AY536408 <i>C.g.</i> Malaysia	AT	<b>T</b> GCACCAGCAATCATCCTT	.....	TAAACTAGAATT	<b>C</b> GACTCAT								
AJ854171 <i>C.g.</i> Malaysia	AT	<b>C</b> GCACCAGCAATCATCCTT	.....	TAAACTAGAATT	<b>C</b> GACTCAT								
EF092290 <i>C.g.</i> Thailand	AT	<b>T</b> GCACCAGCAATCATCCTT	.....	TAAACTAGAATT	<b>C</b> GACTCAT								
DQ923416 <i>C.g.</i> Malaysia	AT	<b>T</b> GCACCAGCAATCATCCTT	.....	TAAACTAGAATT	<b>C</b> GACTCAT								
DQ923417 <i>C.g.</i> Malaysia	AT	<b>T</b> GCACCAGCAATCATCCTT	.....	TAAACTAGAATT	<b>C</b> GACTCAT								
DQ923418 <i>C.g.</i> Puerto Rico	AT	<b>T</b> GCACCAGCAATCATCCTT	.....	TAAACTAGAATT	<b>C</b> GACTCAT								
EF092291 <i>C.g.</i> U.S.A.	AT	<b>T</b> GCACCAGCAATCATCCTT	.....	TAAACTAGAATT	<b>C</b> GACTCAT								
AY683220 <i>C.f.</i>	AT	<b>T</b> GCACCAGCAATCATCCTT	.....	AAAATTAGAATT	<b>C</b> GACTCAT								
Amino acid	I	A	P	A	I	I	L	.....	E	F	D	S	.

**Note:** Bases are numbered relative to the COII gene. Transition mutations are indicated in boldface, one in position 201 of the COII gene and the other in position 354 of the COII gene. These sequence changes do not result in amino acid changes. *C.c.*, *Cornitermes cumulans*; *C.b.*, *Cornitermes bequaerti*; *C.g.*, *Coptotermes gestroi*; *C.f.*, *Coptotermes formosanus*.

The data also suggest that two distinct introductions may have occurred in North America, as suggested by Jenkins et al. (2007): one in the urban area of Key West, Florida (USA) and one in Cleveland, Ohio (USA). These *C. gestroi* populations share the same mutation with populations in Brazil and populations from some regions of Malaysia (a transition in position 201 of the COII gene; see Table 3). In a phylogeographic study using 16S rRNA and COII genes and an internal transcribed spacer, Jenkins et al. (2007) found that *C. gestroi* of Singapore and Ohio are closely related and that samples from Australia, Puerto Rico, and Key West (Florida) are more closely related to samples from Malaysia. Our data support the conclusion of Jenkins et al. (2007), as the populations of Ohio and Florida are clearly clustered in two separate clades in our phylogenetic analysis.

Comparison of the outgroup *Cornitermes cumulans* (Termitidae: Nasutitermitinae) with *C. gestroi* and *C. formosanus* (Rhinotermitidae) shows at position 201 of the COII gene a nucleotide with a T base and at position 354 a base C, thus suggesting that these conditions may be ancestral for both families.

A lack of variation in mtDNA sequences of introduced species of termites in regions outside their natural area of occurrence is not uncommon. Using RFLP techniques, Broughton and Grace (1994) showed low genetic variation (mtDNA) in 10 populations of another exotic *Coptotermes* species, namely *C. formosanus* introduced in Hawaii, from two regions of the state. According to these authors, the low level of mtDNA variation of Hawaiian populations may be due to colonization by a small number of individuals that arrived on ships carrying infested wood or wood products.

Fontes and Milano (2002) calculated a dispersal rate through swarming of 250–300 linear metres per year from the area infested by *C. gestroi* in the large city of São Paulo. The huge swarming, the easy installation of incipient colonies in cargos transported by ship or road, the recent spread (in the last two or three decades) to distant points in Brazil, and the possible single original introduction supported by our genetic study all indicate the potential risk of this exotic subterranean termite dispersing over the continent by means of international commerce.

Complementary molecular studies, such as microsatellite analysis and restriction fragment analysis, are desirable to corroborate our findings and improve the analysis.

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